NUCLEIC ACID DETECTION

BACKGROUND

The present invention relates to detection of a nucleic acid sequence in a mixture of different nucleic acids and a kit therefor.

Widespread conventional techniques for the detection of nucleic acid sequences are the southern blotting technique for DNA and the northern blotting technique for RNA. At the beginning of these procedures the nucleic acid mixtures are separated in nucleic acids of different mass using gel electrophoresis, for example in an agarose or polyacrylamide gel. Following the gel electrophoresis, the different nucleic acids are preferably converted to single stranded nucleic acids. The single stranded nucleic acids are then transferred onto a microcellulose or a nylon filter and are crosslinked with the membrane using heat or UV radiation. The membrane is then blocked with a blocking reagent to saturize all unspecific binding sites of the membrane. Subsequently the nucleic acids fixed on the membrane are hybridized with a labeled nucleic acid probe, which includes a primary sequence complementary to the primary sequence of a target nucleic acid sequence. The label of the nucleic acid probe often contains ^{32}P-labeled phosphates, which can be detected due to their radioactivity (see for example Figure 1). The northern or southern blotting techniques therefore involve lots of different steps, e.g. gel electrophoresis, blotting onto a membrane and detection by hybridization, which are very time consuming and complicated to carry out. For the southern and northern blotting techniques different media (gels for gel electrophoresis and membranes for the blotting) are used, so that lots of different and at least partially expensive materials are used.

DISCLOSURE

Therefore there is a need for a new method for detection of a target nucleic acid sequence in a mixture of different nucleic acids, which allows a fast and reliable detection of a target nucleic acid without the necessity to use blotting techniques. The present invention meets these needs by providing a method for detection of a target nucleic acid sequence according to the base claim 1. Favorable embodiments of the method of the invention and a kit for the detection of the target nucleic acid sequence are subjects of further claims.

Embodiments of the invention provide a fast and easy-to-handle procedure for detection of a target nucleic acid sequence, wherein the hybridizing of the target nucleic acid sequence with the probe takes place in liquid phase. The procedure in A) therefore avoids the complicated, time-consuming and also material-consuming stepproceedure of transfer of the nucleic acids onto a membrane. Furthermore an operator carrying out the embodiments of the invention usually needs less skill than an operator carrying out conventional Northern or Southern blot techniques. After the hybridizing of the target nucleic acid sequence with the labeled probe in stepA), forming a at least partially double stranded hybrid strand between the target nucleic acid and the probe, the different nucleic acids and the target nucleic acid sequence are separated in subsequent B), allowing a detection of the target nucleic acid sequence in following C). Therefore the method for detection requires the hybridizing of the probe with the target nucleic acid sequence prior to separating the nucleic acids. This sequence of the method is reversed in comparison to the conventional northern and southern blotting techniques, where the nucleic acids are separated first and then hybridized with a labeled probe.

In A1), the additional binding sites are hybridized with single-stranded nucleic acids having random primary sequences in liquid phase. The additional binding sites of the nucleic acids, which are still present after A) are often comprised of unpaired bases in single stranded areas of the nucleic acids.

The single-stranded nucleic acids can basepair with single-stranded parts of the

different nucleic acids in the nucleic acid mixture and if present also with single-stranded parts of the target nucleic acid sequence, forming nucleic acid double strands. Therefore after A1) the nucleic acids in the nucleic acid mixture are mainly double-stranded, simplifying the separation of the different nucleic acids in the subsequent B). Due to A1) no retardation of the double stranded hybrid between the probe and the target nucleic acid sequence in comparison to the other nucleic acids occurs during the separation procedure in B). A retardation of the double stranded hybrid during gel electrophoresis normally takes place, when single stranded nucleic acids are still present in the nucleic acid mixture and are also subjected to gel electrophoresis, so that the important information about the size of the target sequence is lost. In practice the information about the size of the target sequence is often used as a control for the correct hybridization between the target sequence and the probe.

Advantageously short nucleic acids with a random primary sequence having a length of 6 to 14 nucleotides are provided in A1) for conversion of the single-stranded parts of the nucleic acid mixture into double-stranded parts. These short oligonucleotides are easy to synthesize and can easily be handled during A1). Due to their small size, these oligonucleotides reliably interact with single-stranded areas in the nucleic acid mixture.

In another variant , the hybridizing in A1) is carried out at roughly room temperature and the hybridizing of the probe with the target sequence in A) is carried out at a temperature between 30°C to 72°C, preferably 56°C to 72°C. A temperature between 30°C to 48°C can also be useful. A further preferred condition for hybridizing in A) is a pH range between 6 to 8.5, preferably slightly alkaline, for example pH 7.5 (e.g. TRIS EDTA buffer pH 7.5).

Due to the low temperature during hybridizing in A1) mismatches in the base pairing do not impair interaction between single-stranded areas of the different nucleic acids in the mixtures and the oligonucleotides with the random primary sequence. In contrast to the low temperature in A1) a higher temperature in A) is used in order to provide a more stringent condition for hybridizing, therefore enabling a good

selectivity during the detection of the target nucleic acid sequence by the probe, reducing false signals.

A nucleic acid having a length of at least 2 times the length of the oligonucleotides with the random primary sequence can be used as a probe. When the probe is large compared to the oligonucleotides with the random sequence, it is possible to carry out A1) and A) simultaneously. Due to co-operative effects, the large probe is still able to interact with the correct target sequence and can also replace short oligonucleotides with the random primary sequence, which already have bound to the target nucleic acid sequence. This variant therefore provides the hybridization of the target nucleic acid sequence with the probe and the conversion of the single-stranded areas of the nucleic acid into double stranded nucleic acids in one go. This procedure therefore reduces the number of method sequences, allowing a faster and easier detection of the target nucleic acid sequence.

Advantageously, in A1) nucleic acids labeled with a second label are used for hybridizing, the second label being different from the first label.

Due to the different labels for the probe and for the nucleic acids having random primary sequences, the amount and the size of the target nucleic acid sequence and the amount of the total nucleic acids in the mixture can be determined using different detection methods.

It is also possible that the nucleic acids with the random primary sequence used for hybridizing in A1) are labeled with a second label after A1), the second again being different from the first label. Such a subsequent labeling of the nucleic acids can, for example, be carried out using dyes like ethidiumbromide, acridine orange, proflavin or Sybr Green®. These intercalating agents are normally used to stain double- or single-stranded nucleic acids.

It is also possible to label the oligonucleotides with the random primary sequence used in A1) by a random-oligonucleotide labeling method, developed by Feinberg and Vogelstein (Feinberg, A.P., Vogelstein, B., Anal Biochem 137, 266 - 267, 1984). Using this method random decanucleotide primers can be used for synthesis of

complementary strands of template nucleic acids. The complementary strands are synthesized from the 3'-end of the random decanucleotide primers using, for example Klenow fragment of DNA polymerase I. In the presence of nucleotides, which are marked with a label, for example biotine or ^{32}P, labeled oligonucleotides for A1) are synthesized.

Favorably, in A2) prior to A) the mixture of different nucleic acids is denatured.

Denaturing advantageously converts the nucleic acids, which might be double-stranded into single-stranded nucleic acids, so that the hybridization in subsequent A) can occur without major difficulties. Denaturing might be carried out, for example, by heating the nucleic acid mixture to high temperatures, for example 90°C to 99°C, preferably 95°C to 99°C for a certain time, e.g. five minutes and immediately reducing the temperature afterwards e.g. by chilling on ice.

Preferably in A) a nucleic acid is used as a probe, having a stretch of 18 to 25 nucleotides being able to hybridize with the target nucleic acid sequence, this stretch having at least 80% sequence homology to the complementary sequence of the target nucleic acid sequence. Alternatively the nucleic acid probe can have at least 12 continuos nucleotides, complementary to the target nucleic acid sequence in order to ensure a good and reliable hybridization between the target nucleic acid sequence and the probe. Such nucleic acid probes can selectively detect the target nucleic acid even within a mixture of other different nucleic acids.

In another embodiment the nucleic acids are separated according to their mass in B) by using a gel electrophoresis. The gel electrophoresis can, for example, be carried out in a polyacrylamide gel or an agarose gel. This separation technique is especially suited to separate the nucleic acids in a reliable manner and in a short time.

Preferably in B) a microfluidic chip having capillaries suitable for nucleic acids electrophoresis is used for separation. The microfluidic chip can comprise, for example, a glass or silicon chip in which capillaries are etched. The capillaries can be filled with a electrophoresis medium, for example polyacrylamide or agarose and

the nucleic acid mixture can be driven through the capillaries using electrophoretic and electro-osmotic forces. Using these microfluidic chips, small volumes can be analyzed very quickly. Therefore microfluidic chips are well-suited to save working time and also reduce the expenses for material.

Preferably the first and the second label are being selected from the following group:

- radioactive labels, fluorescent markers, chemoluminescence, bioluminescence, magnetic labels and antigen labels.

These kind of labels are especially suited to label nucleic acids and can easily be monitored using standard detection methods like autoradiography, fluorescence assays or antibodies.

Preferably fluorescent markers are used as the first and if present second label, wherein the fluorescent markers of the first and second label emit radiation of different wavelengths. Using this variant the detection of both the target nucleic acid sequence and the other different nucleic acids in the mixture can easily by carried out.

When fluorescent markers are used as the first and second label, both fluorescent markers emitting radiation of different wavelengths, the amount and the size of the target nucleic acid and the amount of the other different nucleic acids in the mixture can be determined via the first and second label using a spectrometer for the detection of both labels in C). This embodiment allows a simultaneous detection of both the target nucleic acid sequence and the other nucleic acids in the mixture by simply using a spectrometer e.g. a bioanalyzer instrument.

A person of ordinary skill in the art can synthesize different kinds of nucleic acid probes, depending on the target nucleic acid sequence, which has to be detected. If for example the HI-virus has to be detected in a human tissue sample, a nucleic acid probe can be designed by a person of ordinary skill in the art, which shows a high degree of complementarity in a well-conserved stretch of the HIV genome. This nucleic acid probe might still allow some mismatches in the base pairing, for

example in regions of high variability within different HIV subtypes in order to allow a detection of HIV independent from its subtypes. Furthermore, additional nucleic acid probes for HIV detection can be designed by a person of ordinary skill in the art, having a high degree of complementarity even in regions of high variability in the HIV genome, therefore allowing to distinguish different subtypes of HIV.

In the following embodiments of the invention will be explained in more detail. All figures are just simplified schematic representations presented for illustration purposes only.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the course of separation and detection of a target nucleic acid sequence during a standard northern or southern blotting technique.

The Figures 2 and 3 depict a schematic course of subsequent method proceedure during different embodiments of the invention.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

Referring to Figure 1 the course of subsequent method steps of a conventional southern or northern blot is shown from left to right. At the beginning of a standard detection method for nucleic acids, the nucleic acids are separated in the line 100 of the gel 50 by gel electrophoresis (shown on the left side of the page). A DNA ladder 60 might simultaneously be applied on the gel in line 110 in order to simplify the determination of the size of the nucleic acid in the mixture. Normally only highly abundant nucleic acids are visible after staining, e. g. with ethidiumbromide, like the two bands 70, representing ribosomal RNA. After the separation of the nucleic acids the nucleic acids are transferred onto a nitrocellulose or a nylon filter 80 and are cross-linked with the membrane, as shown in the middle of Fig. 1. The transfer normally also involves the treatment of the gel with NaOH in order to convert the double stranded nucleic acids into single-stranded nucleic acids, which able to hybridize with a nucleic acid probe. The transfer of the nucleic acids from the gel onto the membrane is normally very time-consuming and also requires lots of

material, for example buffer solutions. After transfer, the membrane with the single-stranded nucleic acids is frequently blocked with a blocking reagent in order to saturize all unspecific binding sites on the membrane. This blocking normally takes place by incubating the membrane with commercially available blocking reagents, e. g. Denharts solution, non-fat milk or salmon sperm DNA. Afterwards the membrane is brought into contact with a solution containing a nucleic acid probe 15 with a label 20, as shown on the left side. Normally a ^{32}P-label is used for northern or southern blotting techniques. The labeled nucleic acid probe can hybridize with the target nucleic acid sequence, the membrane is washed and the signal is detected, e. g. by autoradiography.

Turning now to Figure 2, one embodiment is shown. The subsequent procesing of the method is depicted in Figure 2 from left to right. At the beginning a nucleic acid mixture is used, containing different double-stranded nucleic acids 5 and also a double-stranded nucleic acid containing the target nucleic acid sequence 1A and its complementary sequence 1B, both shown in boldfaced representation. In A2) the double-stranded nucleic acids in the nucleic acid mixture are converted to single-stranded nucleic acids, for example by heating the nucleic acid mixture to a high temperature for a short time (for example 95°C for five minutes). Afterwards the nucleic acid mixture contains mainly single-stranded nucleic acids and also the single-stranded target nucleic acid sequence 1A. Subsequently a single-stranded nucleic acid probe 15 with a first label 20 is added in A) and the probe 15 can hybridize with the single-stranded target nucleic acid 1A to form a hybrid between the probe 15 and the target nucleic acid sequence 1A. The major advantage of this embodiment in comparison to conventional methods is that A2) and A) are carried out in liquid phase and are therefore much easier to perform than the standard blotting transfer techniques shown in Figure 1. Subsequently the nucleic acid mixture can be separated, for example in a gel 50 in B) by gel electrophoresis. During C) the hybrid 1A, 15 between the target nucleic acid sequence 1A and the probe 15 can be detected, for example by using a spectrometer with the wavelength λ_1 if a fluorescent marker is used as a label. In this case the nucleic acid band containing the hybrid 1A, 15 lights up and can therefore be detected.

The denaturing of the nucleic acid mixture in A2) is compulsory, when a double stranded DNA target nucleic acid sequence has to be detected within a mixture of other double-stranded DNA molecules. It can readily be seen in Fig. 2, that the separation of the nucleic acid mixture in B) and the detection of the target nucleic acid sequence in C) are both carried out in the gel 50, so that no transfer onto a membrane is necessary.

Referring now to Figure 3 another embodiment is shown from left to right. In this case the nucleic acid mixture contains single-stranded nucleic acids 5 having additional binding sites 10 and also a single-stranded target nucleic acid sequence 1A, shown again in boldfaced representation. In other cases it might be preferred to denature even single-stranded nucleic acid mixtures in order to ensure a good base pairing between the probe and the target nucleic acid sequence. In A) of this embodiment the single-stranded target nucleic acid sequence 1A is hybridized with the nucleic acid probe 15, which is labeled with a first label 20. In subsequent A1) oligonucleotides 25 with a random primary sequence, having a second label 30 are incubated with the single-stranded nucleic acids 5 in the mixture in order to bind to the additional binding sites 10, thereby converting nearly all single-stranded nucleic acids 5 into double-stranded nucleic acids. During A1) multiple oligonucleotides 25 can bind to one single-stranded nucleic acid 5 converting this nucleic acid into a double-stranded form. Additionally the oligonucleotides 25 might also bind to single-stranded regions of the hybrid between the probe 15 and the single-stranded nucleic acid target sequence 1A. A1) converts almost all of the single-stranded nucleic acids into double-stranded nucleic acids, allowing a precise mass-dependent separation of the double-stranded nucleic acids in subsequent B). The separation again might be carried out in a gel 50. Due to the conversion of A1) no shift of the signal band comprising the hybrid 1A, 25, 15 occurs during the separation of the nucleic acids in B), allowing the determination of the size of the target nucleic acid sequence. If different fluorescent markers are used as the first label 20 and the second label 30 the hybrid between the target nucleic acid sequence 1A, the probe 15 and the oligonucleotides 25 might be simultaneously detected with the other nucleic acids 5 by using a spectrometer with different wavelengths λ_1 and λ_2 in C). This special

embodiment allows the determination of the amount and the size of the target nucleic acid sequence as well as the determination of the amount of the other nucleic acids 5 in the nucleic acid mixture.

EMBODIMENTS

In order to test the feasibility of embodiments of the invention a detection was carried out, detecting the gene for the human glycerin aldehyde phosphate dehydrogenase (GADPH) in human female blood total DNA.

At the beginning the human female blood total DNA was digested using the restriction enzyme Dra I. Afterwards the DNA was concentrated by using a sodium acetate precipitation. 15 µl of sodium acetate 5 M and 175 µl ethanol were added and mixed. Afterwards the DNA was precipitated by incubating the mixture for one hour on ice. The DNA was pelleted by centrifuging 50 minutes at full speed and the DNA pellet was washed in 70% ethanol, dried and resuspended in 5 µl TE buffer (10 mM TRIS, 0.01 mM EDTA). Subsequently the digested DNA was denatured in A2) in order to convert the DNA molecules into single stranded nucleic acids by heating at 99°C for 5 minutes. Then the mixture was chilled on ice. The probe for the GADPH gene, which was labeled with the fluorescent dye BODIPY® 650/665 (available from molecular probes) and decamers with random primary sequence (available from Ambion) were both denatured in separate tubes by heating at 99°C for 5 minutes and chilling on ice. Subsequently A) was carried out by mixing the human female blood total DNA and the labeled probe, incubating for 5 minutes at 99°C, cooling down to 65°C and incubating for five minutes at 65°C. Afterwards the mixture was chilled on ice. Subsequently the decamers with the random primary sequence were added and incubated with the DNA and the labeled probe for five minutes and put on ice again in A1). The labeled GADPH probe consisted of a mixture of different probes having a medium size of 200 to 500 nucleotides, mostly being complementary to the GADPH gene and spanning the whole gene. The probes were synthesized by the random priming reaction of Feinberg and Vogelstein by using hexanucleotides as random primers. After A1) the separation of the nucleic acids in B) was carried out by transferring the nucleic acid mixture onto a DNA 12000

microfluidic chip (Agilent Technologies, Waldbronn, Germany) with 20 µM SYTO 16® (Molecular probes, Eugene, OR, USA) as a nucleic acid specific dye in the gel matrix of the chip as a second label. Using a spectrometer signals for the hybrid between the probe and the target nucleic acid sequence as well as signals for the other nucleic acids could be determined.

The scope of the invention is not limited to the embodiments shown in the figures. Indeed, variations especially concerning the combination of the different optional method features and variations concerning the design of the nucleic acid probe are possible.